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Armed Forces Radiobiology
Research Institute
8901 Rockville Pike
Bethesda, MD, 20889

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Address of Sponsoring Institution 4301 Jones Bridge Road
Bethesda MD 20814

USU Grant Number HU0001-14-1-TS10

USU Project Number N14-P15

Title of Research Study or Evidence-Based Practice (EBP) Project Impact of embedded military metal alloys on skeletal physiology in an animal model

Period of Award 09/01/14 – 8/31/16

Applicant Organization Henry Jackson Foundation

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Mentor Signature (if applicable) _____ Date _____

Abstract

Purpose: The purpose of this study is to determine if differences exists across measures of bone serum biomarkers, computed tomography, biomechanical testing and histomorphometry in bone of skeletally mature rats embedded with tungsten/nickel/cobal (WNiCo), tungsten/nickel/iron (WNiFe) and depleted uranium (DU) compared to Tantalum (Ta) control.

Design: An experimental design was used consisting of 3 experimental groups and one control group. Time points of 1 and 3 months were used and a minimum of 8 animals were euthanized at each time point.

Methods: Animals were surgically implanted with a total of 4 pellets, 2 in each gastrocnemius muscle. After euthanasia, whole blood, serum, and the long bones of the hindquarters were extracted to be used for analysis. Skeletal tissue was analyzed on the whole organ, tissue and cellular level.

Sample: 74 skeletally mature (aged 60-months) Sprague-Dawley rats

Analysis: Results were analyzed using descriptive statistics and one-way analysis of variance. All results were completed on Prism (GraphPad) version 6. Statistical significance was set at $p<0.05$. Tissue level results, correction for body weight was completed to account for additional load bearing by heavier animals.

Findings: Measures at the organ level (serum biomarkers) demonstrated no significant differences however tissue level measures (μCT, pQCT) had significantly increase measures of cancellous bone mineral density and bone mineral concentration as well as cortical bone mineral density in both the WNiCo and DU groups at 3 months. Despite these changes, no change in the physical strength were identified through biomechanical testing.

Implications for Military Nursing: Military nurses are in the unique position to both advocate and educate for any patients who identify themselves as having embedded fragments. Many of these patients will have fragments come out of their skin through natural migration and very little is known about what to do when this happens. Rapid identification of the alloy can be completed and the patient will be followed by systems that are in place through the Veteran's Affairs medical system. Only military nurses understand that embedded metal has become a "normal" finding on many patients who have been in proximity to improvised explosive devices.

TSNRP Research Priorities that Study or Project Addresses

Primary Priority Identify the primary research priority addressed in the study or project.

Force Health Protection:	☐ Fit and ready force ☐ Deploy with and care for the warrior ☒ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other: (specify)	☐

Secondary Priority (if applicable; otherwise delete the words "Secondary Priority" and the duplicate table below)

Force Health Protection:	☐ Fit and ready force ☐ Deploy with and care for the warrior ☐ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☒ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other: (specify)	☐

Progress Towards Achievement of Specific Aims of the Study or Project

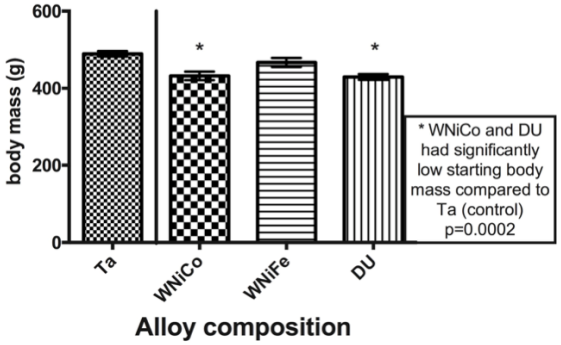
Findings related to each specific aim, research or study questions, and/or hypothesis:

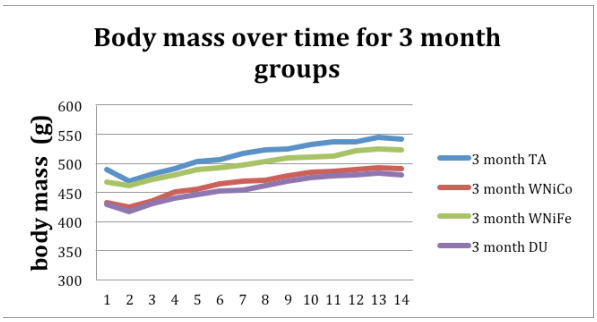
The specific aim of this project is to determine if differences exists across measures of bone serum biomarkers, computed tomography, biomechanical testing and histomorphometry in bone of skeletally mature rats embedded with WNiCo, WNiFe and DU compared to Ta (control).

Animal Weights

Animals were weighed upon arrival to the animal facility, prior to euthanasia and, at a minimum, weekly. Body mass was recorded along with body temperatures as a means of evaluating the health of each animal. A loss of 5% body mass in a week, not proceeded by surgical intervention, would trigger further health evaluation by the primary investigator and the veterinarian. Body mass for 1-month group showed no significant mass differences between groups compared to control at either the arrival to the facility or prior to euthanasia (Figure 2, 3, 4). Additionally, no significant differences exist between any groups.

Body weights in the WNiCo and DU groups were significantly lower upon arrival as well as at euthanasia ($p < 0.0002$). The increase in body weights for each group remained consistent throughout the study (Figure 5, 6, 7). The percent change over time for Ta (control) in the 3-month group is 11%, while WNiCo, WNiFe, and DU are 14%, 12% and 12% respectively.





Pellet mass

Each metal pellet was weighed prior to insertion as well as following extraction. Prior to weighing each pellet after euthanasia, they were soaked in 70% ethanol to ease the removal of any tissue that may have adhered to the pellet during the implantation period. The only pellet that had a significant weight change over time was in both the 1-month and 3-month DU groups (13.4% and 17.1% respectively). All other pellets decreased in mass by 3.5%-6.6%.

Hematology

Hematology analysis was performed on the day of euthanasia. Controls were completed and no sample was evaluated until results of controls were within normal limits.

Complete blood counts (CBC) were completed consisting of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT) and platelet count (PLT). Each sample was evaluated twice to ensure accuracy and decrease the risk of a sampling error. If differences were minimal, less that 10%, then the first sets of results were used. If any 2 results differed by greater than 10%, then a total of 3 evaluations were completed, the two most consistent results were kept and averages between the groups were used as the results.

No significant differences are observed between any experimental groups at the one-month time point and control on measures of CBC. Among the 3-month groups, platelet count is significantly lower in WNiFe compared to control ($p=0.0285$). Though the platelet count was statistically significant between the WNiFE and Ta groups, these results have no clinical significance and do not reflect thrombocytopenia. No other significant differences seen on CBC at 3 months.

Serum Biomarkers

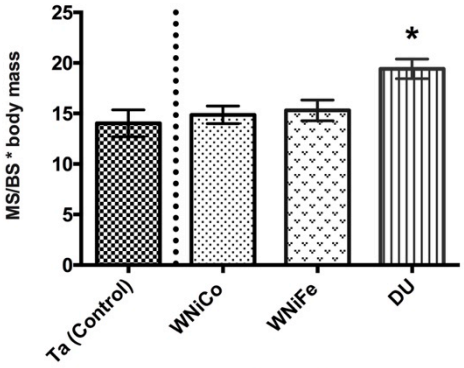
Serum markers of bone turnover were completed and statistical comparison performed for each time point. Each ELISA was performed according to the instructions within each kit. The first two osteocalcin kits yielded no results for either controls or samples. Additional kits were ordered yielding results consistent with expectations for controls. Results of osteocalcin ELISA were evaluated and any results with a coefficient of variation greater than 25% were omitted from analysis. Results for 1-month time point resulted in no significant changes between any groups on measures of osteocalcin (Figure 7). The 3-month time point group demonstrates significantly lower osteocalcin between WNiCo and DU, however, no experimental groups differed from control.

Results of TRAP5b ELISA were evaluated and any results with a coefficient of variation greater than 25% were omitted from analysis. Measures of TRAP5b reveal no significant differences at both the 1 and 3-month timepoints.

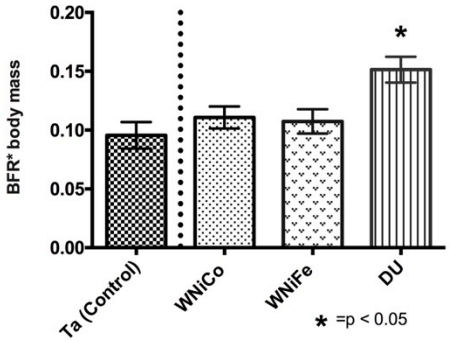
Histomorphometry

Histomorphometry was completed utilizing ultraviolet light in order to visualize the fluorochrome label, calcein. The evaluation was completed using Osteomeasure with 10% of samples being evaluated twice in order to ensure consistency of this subjective measure. Results for mineralized surface/bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR) were completed and analyzed.

No statistically significant differences were noted on measures MAR between any experimental groups and control. Depleted uranium shows a significantly increase in MS/BS at 3 months.

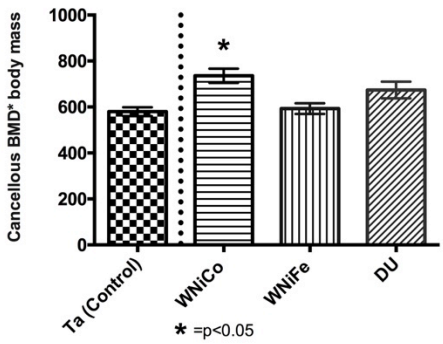


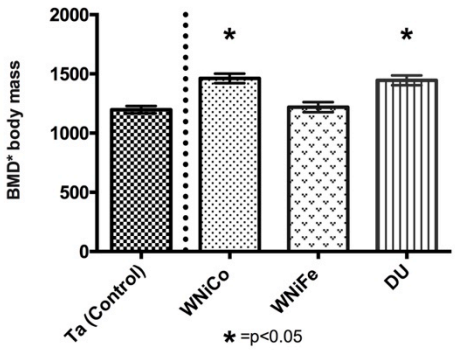
On measures of BFR, no statistically significant differences exist at the 1-month time point between the experimental groups and control. However, at the 3-month time point, BFR is significantly increased in the DU by 43% over control (p=0.0296).



Peripheral quantified computed tomography

Bone mineral density and bone mineral concentration for the trabecular compartment of the distal femur are not significantly different than control at either the 1-month or 3-month time point. On the measure of bone mineral density, while no differences are seen at the 1-month time point, the WNiCo group shows statistically significant (p=0.0163) greater bone mineral density over control. WNiCo and DU have significantly increased total vBMD and WNiCo has increase cancellous BMD compared to control at 3 months





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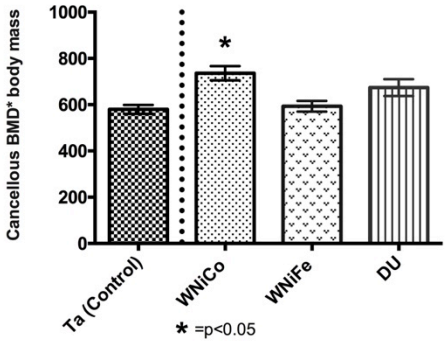
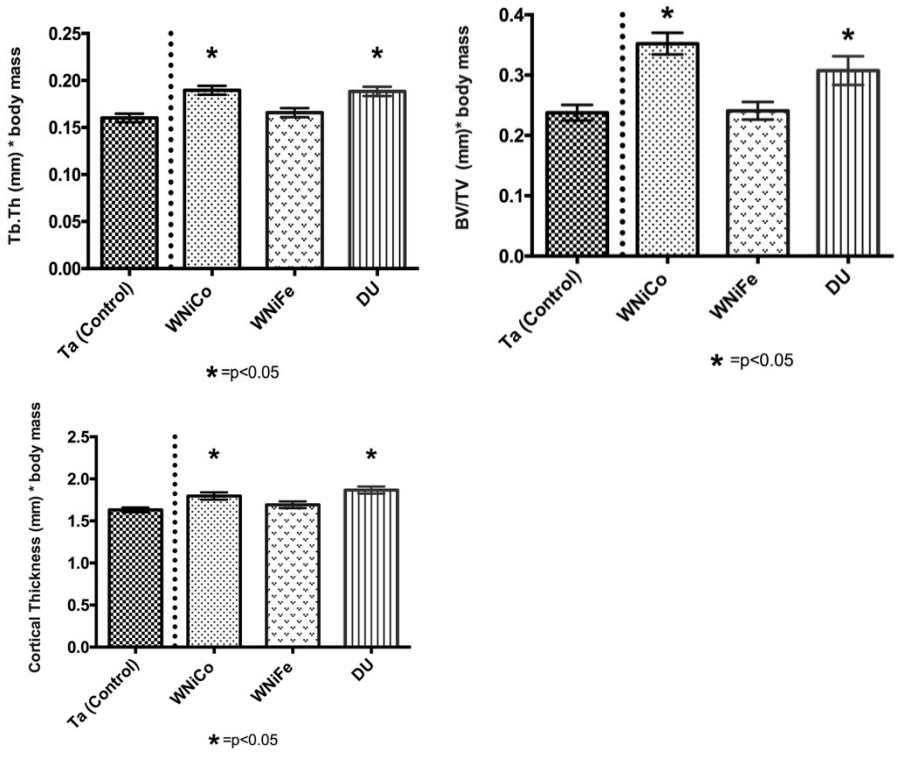


Mid-shaft femur pQCT scans revealed no statistically significant results on cortical thickness at either 1-month or 3-month time points.

Micro computed tomography

Each tibia was x-rayed in order to measure the total length of the bone to be scanned. The range of the total length of each tibia was from 38-44mm. No significant differences were seen on any trabecular measurements at either the 1-month or 3-month time point on measures of trabecular thickness, trabecular spacing, or trabecular number. Cortical thickness measurements at 1-month were not statistically significant however, in the 3-month group, the WNiCo group is significantly increased on the measure of cortical thickness compared to control ($p=0.0015$). WNiCo and DU groups have significantly increased Bone Volume/ Total Volume at 3 months



Biomechanical Testing
Biomechanical testing included cross-sectional moment of inertia, elastic modulus, ultimate stress and energy to yield. No statistically significant differences are seen in either the 1-month or 3-month time points between any of the experimental metal groups and control (Table 16-22). These results indicate that no difference exists between groups implanted with military metal alloys and control when the physical strength of the bone is measured. Regardless of increased cortical bone seen on uCT in the 3-month WNiCo group, no change in strength is present.

Relationship of current findings to previous findings: Previous work in this area suggested that bone loss would occur in the animals implanted with WNiCo and DU. The previous studies utilized the same species of animals and the identical composition of metal, however the animals were not skeletally mature and the theory of bone loss was not substantiated by measure at different levels of organ physiology. Additional studies that examined the effect of these metals on local tissue determined that immediate genotoxic changes occur after implantation and that the formation of metastatic cancer occurs in rats with WNiCo in a very short timeframe. The current findings show that instead of bone loss, there is a relative increase in bone at both the cancellous and cortical levels. This is significant because it is the first time metal has demonstrated bone growth across multiple measures of bone physiology.

Effect of problems or obstacles on the results: No problems or obstacles were encountered during this study that had any effect on the results

Limitations: The greatest limitations to this study are the short time periods of 1 and 3 months as well as the low dose utilized for implantation. With greater time and funding, a lifetime study evaluating varying doses of metals implanted may elucidate on whether the metals are the cause of the changes or if, in fact the immune response plays a role in these changes

Conclusion: The presence of military metal alloys, specifically WNiCo and DU appear to have at least an early effect on bone after 3 months. A greater amount of bone in seen in these groups on measures specific to cancellous and cortical bone which is contrary to previous theories.

Significance of Study or Project Results to Military Nursing

This study demonstrates that, what was once thought of as inert fragments may have an impact on the skeletal health of our service members. Military nurses have the unique position to care for those involved in improvised explosive devices, which often result in embedded fragments being maintained in the body of many of these individuals. These fragments remain in place due to clinical practice guidelines that date back to the civil war. Previous work has demonstrated that cancer can result from these metals. Specific to bone, no known work has been done with these metals up to this date. This study clearly shows that there is an effect by three months on bone from WNiCo and DU. Due to the anabolic effects of cancer and what appears to be bone growth, and increase in bone formation may be detrimental to skeletal health of these individuals.

Changes in Clinical Practice, Leadership, Management, Education, Policy, and/or Military Doctrine that Resulted from Study or Project

Policy implications for this study are currently in place and the VA medical system has already begun to examine the bone density of veterans with known DU exposure. From a clinical practice standpoint, all metals that have been removed from a service member should be evaluated at the Joint Pathology Lab for metal composition. Educating patients and clinicians on the potential impact of this study and the known literature will help change the culture from one of a "war trophy" that was embedded to a potentially toxic substance. This represents a change in mindset across all of DOD and VA, and possibly across all healthcare following the domestic terror events that leave Americans with similar patterns of injury. Though no change has occurred in military doctrine, this study helps solidify that current policy is accurate and should be followed.

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References Cited
Summary of Dissemination

Type of Dissemination	Citation	Date and Source of Approval for Public Release
Publications (using a consistent reference style, provide complete citation for papers already published in print or electronic journals)		
Publications in Press (using a consistent reference style, provide partial citation for papers accepted for publication but not yet published in print or electronic journals; if known, provide estimated date paper will be published)		
Published Abstracts (using a consistent reference style, provide complete citation for abstracts published in print or electronic journals)		
Podium Presentations (using a consistent style, provide author(s), title of presentation, conference name, conference location, date of presentation, sponsoring agency or organization)		
Poster Presentations (using a consistent style, provide author(s), title of		

poster, conference name, conference location, date of presentation, sponsoring agency or organization)		
Media Reports (provide details such as title, type of media [e.g., press release, newspaper article, television or radio story, internet post], date of report)		
Other		

Reportable Outcomes

Carefully document reportable outcomes; add rows to the appropriate categories as needed

Reportable Outcome	Detailed Description
Applied for Patent (if none, type "none")	
Issued a Patent (if none, type "none")	
Developed a cell line (if none, type "none")	
Developed a tissue or serum repository (if none, type "none")	
Developed a data registry (if none, type "none")	

Recruitment and Retention Aspect	Number
Animals Projected in Grant Application	80
Animals Purchased	80
Model Development Animals	0
Research Animals	80
Animals With Complete Data	75
Animals with Incomplete Data	5

Recruitment and Retention Aspect	Number
Animals Projected in Grant Application	80
Animals Purchased	80
Model Development Animals	0
Animals Intervention Group / Control or Sham Group	60/20
Intervention Group / Control or Sham Group Animals With Complete Data	58/17
Intervention Group / Control or Sham Group Animals With Incomplete Data	2/3

Final Budget Report

HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDICINE
Advancing Military Medical Research

TASK BUDGET SUMMARY

Current as of: JAN2017

Organization: HJF-Henry M. Jackson Foundation
Award #/Name: 64442 - METAL ALLOY IMPACT
Award Manager: DANCHANKO, WILLIAM
Award Period: 09/01/2014 to 08/31/2016
Project #/Name: 307382 - METAL ALLOY IMPACT
Project Manager: SHEPARD, ALYSSA
Project Period: 09/01/2014 to 08/31/2016

Task # / Name: 1.00 - TASK 1
Task Period: 09/01/2014 to 08/31/2016
Task Manager: DANCHANKO, WILLIAM
Task Desc: N14-P15
Award Sponser: UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
Billing Analyst: DORSEY, TRACEY M.
Primary Analyst: LIVAN, REYHAN
Ref Award#: HU0001-14-1-TS10

Current As of	Award/Project/Task Number	Task Budgetary Control	Category Group	Expenditure Category	Budgetary Control	Current Month Expenses	Budget	Open Commitment	Task-To-Date Expenses	Total Funds Used	Balance Available	Percentage Available
01/31/2017	64442 - 307382 - 1.00	Absolute	DIRECT	SUPPLIES	Absolute	0.00	37,416.00	0.00	24,136.65	24,136.65	13,279.35	35.49
				DOMESTIC TRAVEL	Absolute	0.00	2,000.00	0.00	1,206.17	1,206.17	793.83	39.69
				OTHER DIRECT COSTS	Absolute	0.00	2,000.00	0.00	642.70	642.70	1,357.30	67.87
				TOTAL DIRECT :		**0.00**	**41,416.00**	**0.00**	**25,985.52**	**25,985.52**	**15,430.48**	**37.26**
			INDIRECT	ON-SITE OVERHEAD	Advisory	0.00	15,108.56	0.00	9,426.30	9,426.30	5,682.26	37.61
				COMPANY-WIDE G & A	Advisory	0.00	8,083.02	0.00	5,063.96	5,063.96	3,019.06	37.35
				TOTAL INDIRECT :		**0.00**	**23,191.58**	**0.00**	**14,490.26**	**14,490.26**	**8,701.32**	**37.52**
				TOTAL TASK :		**0.00**	**64,607.58**	**0.00**	**40,475.78**	**40,475.78**	**24,131.80**	**37.35**

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14. ABSTRACT

Purpose: The purpose of this study is to determine if differences exists across measures of bone serum biomarkers, computed tomography, biomechanical testing and histomorphometry in bone of skeletally mature rats embedded with tungsten/nickel/cobal (WNiCo), tungsten/nickel/iron (WNiFe) and depleted uranium (DU) compared to Tantalum (Ta) control.

Design: An experimental design was used consisting of 3 experimental groups and one control group. Time points of 1 and 3 months were used and a minimum of 8 animals were euthanized at each time point.

Methods: Animals were surgically implanted with a total of 4 pellets, 2 in each gastrocnemius muscle. After euthanasia, whole blood, serum, and the long bones of the hindquarters were extracted to be used for analysis. Skeletal tissue was analyzed on the whole organ, tissue and cellular level.

Sample: 74 skeletally mature (aged 60-months) Sprague-Dawley rats

Analysis: Results were analyzed using descriptive statistics and one-way analysis of variance. All results were completed on Prism (GraphPad) version 6. Statistical significance was set at $p<0.05$. Tissue level results, correction for body weight was completed to account for additional load bearing by heavier animals.

Findings: Measures at the organ level (serum biomarkers) demonstrated no significant differences however tissue level measures (μCT, pQCT) had significantly increase measures of cancellous bone mineral density and bone mineral concentration as well as cortical bone mineral density in both the WNiCo and DU groups at 3 months. Despite these changes, no change in the physical strength were identified through biomechanical testing.

Implications for Military Nursing: Military nurses are in the unique position to both advocate and educate for any patients who identify themselves as having embedded fragments. Many of these patients will have fragments come out of their skin through natural migration and very little is known about what to do when this happens. Rapid identification of the alloy can be completed and the patient will be followed by systems that are in place through the Veteran's Affairs medical system. Only military nurses understand that embedded metal has become a "normal" finding on many patients who have been in proximity to improvised explosive devices.

15. SUBJECT TERMS
Skeletal physiology, Animals, serum biomarkers

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					Jaya Pothen
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